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CHARACTERIZATION OF GREY MATTER ATROPHY FOLLOWING 6-HYDROXYDOPAMINE LESION OF THE NIGROSTRIATAL SYSTEM

Robert Westphal[a1*], Akira Sumiyoshi[c], Camilla Simmons[a], Michel Mesquita[a], Tobias C Wood[a], Steve CR Williams[a], Anthony C. Vernon[b], Diana Cash[a]

[a]Department of Neuroimaging, [b]Department of Basic and Clinical Neuroscience, King's College London, Institute of Psychiatry, Psychology & Neuroscience, UK

[c]Institute of Development, Ageing and Cancer, Tohoku University, Japan

*Corresponding author

Email: robert.westphal@psych.ox.ac.uk Phone: 00491865613111

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[1] Permanent Address: Department of Psychiatry; Warneford Hospital; University of Oxford; Warneford Lane; Oxford OX3 7JX; United Kingdom

Abstract

Background: The unilaterally-lesioned 6-hydroxydopamine (6-OHDA) rat is one of the most commonly used experimental models of Parkinson's disease (PD). Here we investigated whether magnetic resonance imaging (MRI) that is widely used in human PD research, has the potential to non-invasively detect macroscopic structural brain changes in the 6-OHDA rat in ways translatable to humans.

Methods: We measured the grey matter composition in the unilateral 6-OHDA rat in comparison to sham animals using whole-brain voxel-based morphometry (VBM) - an unbiased MR image analysis technique. The number of nigral dopamine neurons and the density of their cortical projections were examined *post-mortem* using immunohistochemistry.

Results: VBM revealed widespread bilateral changes in grey matter volume on a topographic scale in the brains of 6-OHDA rats, compared to sham-operated rats. The greatest changes were in the lesioned hemisphere, which displayed reductions of grey matter volume in motor, cingulate and somatosensory cortex. Histopathological results revealed dopaminergic cell loss in the substantia nigra and a denervation in the striatum, as well as in the frontal, somatosensory and cingulate cortices.

Conclusion: Unilateral nigrostriatal 6-OHDA lesioning leads to widespread grey matter volume changes, which extend beyond the nigrostriatal system and resemble advanced Parkinsonism. This study highlights the potential of structural MRI, and VBM in particular, for the system-level phenotyping of rodent models of Parkinsonism and provides a methodological framework for future studies in novel rodent models as they become available to the research community.

Abbreviations

6-OHDA, 6-hydroxydopamine; ANOVA, analysis of variance; CSF, cerebrospinal fluid; DA, dopamine; DAB, diaminobenzidine; DARTEL, diffeomorphic anatomical registration using exponentiated lie algebra; FWER, family-wise error rate; GM, grey matter; GMV, grey matter volume; MFB, medial forebrain bundle; MRI, magnetic resonance imaging; PD, Parkinson's disease; PST, population-specific template; ROI, region(s) of interest; SEM, standard error of the mean; T2W, T2-weighted; TBS, Tris-buffered saline; TH, tyrosine hydroxylase; TH+, tyrosine hydroxylase positive; VBM, voxel-based morphometry; WM, white matter; WMV, white matter volume

Introduction

Parkinson's disease (PD) is a multi-system, progressive neurodegenerative movement disorder (Lees et al., 2009). Pathological hallmarks of the disease include degeneration of dopamine (DA) neurons in the substantia nigra (SN) pars compacta and the presence of alpha-synuclein immunopositive Lewy bodies in the surviving DA neurons (Spillantini et al., 1997, Lees et al., 2009). Currently there are no treatments that can slow, halt or reverse the progressive neurodegeneration that occurs in PD (Stocchi and Olanow, 2013). Addressing this unmet medical need requires the generation of animal models that show improved construct, face and predictive validity to the human disorder (see (Duty and Jenner, 2011) for review)). The development of reliable and validated cross-species biomarkers for the non-invasive detection and longitudinal monitoring of neuropathological changes in rodent models, in a clinically comparable manner, would facilitate this process. For example, such markers would have the potential to be proxy measures of the efficacy of novel therapeutic interventions, particularly those with disease modifying potential. Magnetic resonance imaging (MRI) is well suited to this task. It is widely available, relatively inexpensive and mostly non-invasive, with excellent soft-tissue contrast and spatial resolution, permitting the detailed macro- and microscopic measurements of dynamic structural and functional parameters in both human and animal subjects (Finlay et al., 2014). In this context, there is an extensive literature on the use of structural MRI and computational image processing methods, particularly voxel-based morphometry (VBM) (Burton et al., 2004, Nagano-Saito et al., 2005, Summerfield et al., 2005, Song et al., 2011, Weintraub et al., 2011, Hattori et al., 2012, Menke et al., 2013) to map the spatiotemporal sequence of topographical brain changes in Parkinsonism and related disorders. Overall, these data show that cortical atrophy in prefrontal, temporal, occipital and parietal cortices can be generally observed even in early stage PD, but is more pronounced in advanced PD, specifically in the temporal brain areas in PD patients with dementia.

Despite this, MRI-based measures are yet to be adopted as primary outcomes in clinical trials in the assessment of novel therapeutics and the utility of structural MRI as a true biomarker for PD remains somewhat unclear. One obstacle is the inconsistency between structural imaging studies, which is likely caused by multiple factors such as differences in the number of subjects, duration of the disease, the heterogeneity of Parkinsonism and the use of different image analysis methods in different centres. In addition, human MRI studies cannot

(to date) identify the cellular mechanisms that drive structural brain changes (particularly grey matter volume (GMV) and white matter volume changes) including whether these are specific to defined populations of vulnerable neurons – in this case, the A9 DA neurons in the SN. Together, these represent a significant barrier to the use of routine anatomical MRI to track disease progression and neuroprotective efficacy of novel (or repurposed) drugs in PD patients.

To address these issues, one fruitful approach may be to phenotype rodent models of Parkinsonism, in which genetic and environmental variables may be precisely controlled, with structural MRI (clinically-comparable technology) and computational image analysis tools relevant to human MR imaging to permit parallel processing. The unique advantage of this approach is that the spatiotemporal sequence of brain volume changes may be identified and then linked to *post-mortem* changes at the cellular and molecular level to identify potential underlying mechanisms that explain the same MR measurements in humans (Finlay et al., 2014). Proof-of-concept for this approach is provided by prior studies in which longitudinal MRI was utilised to identify patterns of brain atrophy in rodents rendered hemi-parkinsonian following micro-injection of the proteasome inhibitor lactacystin into the nigrostriatal system (Vernon et al., 2010, Vernon et al., 2011, Harrison et al., 2015). Using deformation based morphometry these data replicated patterns of grey matter (GM) atrophy apparent in end-stage PD patients with co-morbid dementia, including decreased volume of the putamen and widespread cortical regions, which did not appear to be driven by neuronal loss (Vernon et al., 2011). Interestingly, deformation based morphometry data also suggested GM atrophy of the ventral midbrain and thalamus, both of which were related to neuronal loss. Crucially however, whilst dopaminergic neurons may be preferentially sensitive to proteasome inhibition (McNaught et al., 2002) there is also evidence to suggest that synthetic proteasome inhibitors induce dose-dependent dopaminergic neuronal degeneration and are associated with a significant risk of non-specific neuronal and/or glial cell toxicity at higher doses (Vernon et al., 2010, Xie et al., 2010). Thus, whilst the MRI changes observed in these prior studies are clearly linked to DA depletion, the possibility that these may also reflect additional loss of other vulnerable neuronal or glial cell populations cannot be entirely excluded. Although this is not necessarily a limitation, since PD is clearly a multi-system disorder, it raises the question as to what the pattern of atrophy would be following specific and selective lesions of nigral dopaminergic neurons. Specifically, if brain structural changes

in such a model would be similar or different to both those found in the lactacystin rat and importantly, in clinical patients.

We therefore sought to address this issue by providing a methodological framework for developing structural MRI biomarkers following a selective lesion of catecholaminergic neurons. Specifically, we collected structural MRI data from rodents lesioned with the catecholamine-neuron selective neurotoxin 6-hydroxydopamine (6-OHDA), the prototypical rat PD model (Ungerstedt, 1968). We analysed the MRI data using a recently-developed VBM procedure in rats that uses clinically relevant algorithms for the translational validation of pathoanatomical changes observed in humans (Suzuki et al., 2013). We also tested the hypothesis that the loss of DA neuron fibres may be a more prominent driver of extra-nigral atrophy in the cortex and striatum, since prior work in the lactacystin model found no change in neuronal number in these regions (Vernon et al., 2011). We therefore examined the pattern of tyrosine hydroxylase positive (TH+) fibre denervation post-mortem in the cortex of 6-OHDA lesioned rats and sham controls. We hypothesised that the MRI phenotype of the classic 6-OHDA model resembles those seen in lactacystin-lesioned rats and in advanced clinical idiopathic Parkinson's disease.

Experimental procedures

Animals

Adult male Sprague-Dawley rats (N=28; 260 ± 25g, Harlan, UK) were group-housed at 21±1°C in a 12 hour light:dark cycle and with *ad libitum* access to standard rat chow and drinking water. All experiments were conducted in accordance with the Home Office Animals (Scientific procedures) Act, UK, 1986 and were approved by the King's College London ethical review committee.

Unilateral 6-OHDA lesioning procedure

Rats were divided into a sham-operated (N=12) or 6-OHDA lesioned group (N=16). Animals were anesthetized (isoflurane 2.5% in oxygen/air 1:4, 1 l/min, Baxter International Inc., Deerfield, IL, USA) and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA, USA). Stereotaxic intracerebral injections of 6-OHDA, or saline (control) were performed using a Hamilton syringe (Hamilton, Reno, NV, USA) and motorized syringe pump (Harvard Apparatus, Holliston, MA, USA). A severe lesion of the nigrostriatal pathway was obtained by injecting 12 µg 6-OHDA (Sigma-Aldrich, St. Louis, MO, USA) dissolved in 4 µl sterile saline (Sigma-Aldrich) containing 0.02% (w/v) ascorbic acid (Sigma-Aldrich) into the left medial forebrain bundle (MFB) at coordinates (mm) AP -4.4, ML 1 and -7.8 from dura, according to the rat stereotaxic atlas (Paxinos and Watson, 1997). Sham-operated animals underwent the same procedure but received 4 µl sterile saline only.

Behavioural testing: Apomorphine-induced rotation test

Two weeks post-surgery, the extent of the 6-OHDA lesioning was confirmed by the apomorphine-induced rotational test (Ungerstedt and Arbuthnott, 1970) as described previously (Vernon et al., 2011). Briefly, rats were placed in an automated rotameter and baseline activity recorded for 10 minutes. Rats then received a subcutaneous injection of apomorphine (0.1 mg/kg in 0.9% saline, E-Biomed GmbH, Heidelberg, Germany) and the number of ipsiversive and contraversive full body turns were recorded automatically over a period of 60 minutes. A cut-off of >100 net contraversive turns following apomorphine

challenge was utilised to confirm a significant lesion of nigral dopamine neurons (Papa et al., 1994, Marin et al., 2007, Jang et al., 2012). Three 6-OHDA rats did not meet this rotation criteria following apomorphine challenge and were excluded from further analysis. Thus, N=13 6-OHDA-lesioned rats underwent MR imaging.

Magnetic resonance imaging – Preparation and acquisition

Structural MRI was performed three weeks (±2 days) post-surgery using a 7T MRI scanner (Agilent Technologies, Santa Clara, CA, USA) employing a custom-made birdcage quadrature radiofrequency head coil (72 mm diameter; Rapid Biomedical GmbH, Rimpar, Germany). This time point was chosen to avoid the well-described hyperintense areas on T2-weighted (T2W) images observed in 6-OHDA rats close to the injection site which persist until 14 days post-lesioning (Dhawan et al., 1998, Kondoh et al., 2005). Animals were placed in the centre of the scanner, secured in a head frame and anaesthetized with isoflurane (~2% in 70/30 medical oxygen/air mixture delivered at 1 l/min) throughout the imaging procedure. Respiration, pulse and blood oxygenation were continuously monitored for the duration of the scan (SA instruments, Stony Brook, NY, USA). Body temperature was maintained at 37±1°C using a rectal probe and a thermostat-controlled air heating unit (SA instruments). Anatomical images were acquired using a 2D fast spin echo T2W pulse sequence with following parameters: 40 x 0.5 mm coronal slices, TEeff 60 ms, TR 4000 ms, flip angle 90°, averages 8, field of view 32 x 32 mm, matrix 128 x 256, giving an in-plane resolution of 0.25x0.125 mm, with a total acquisition time of 17 min. In the 6-OHDA group, three rats died after the image acquisition leaving N=13 6-OHDA rats for image analysis, and N=10 for histology. One sham rat was excluded from image analysis due to image artifacts leaving N=11 sham rats for image analysis, and N=12 for histology.

Voxel-based morphometry

To detect and analyse structural brain changes we applied VBM, an automated whole brain morphometry technique that allows automated evaluation of the grey matter composition of 6-OHDA rats in comparison to shams. The VBM method described here follows the procedure described by Ashburner and Friston (Ashburner and Friston, 2000) with modifications for rat MRI datasets as reported previously (Suzuki et al., 2013). The T2W

images were processed and analysed using statistical parametric mapping software (SPM8, http://www.fil.ion.ucl.ac.uk/spm/, Wellcome Department of Cognitive Neurology, London, UK) and FMRIB Software Library (FSL v5.0; http://www.fmrib.ox.ac.uk, Analysis Group, FMRIB, Oxford, UK) and custom-written scripts in MATLAB (MathWorks Inc., Natick, MA, USA). Statistical parameter maps were visualized using MRIcroGL (v1.0; (Rorden and Brett, 2000)).

T2W anatomical images from each rat were first subjected to unified segmentation tool in SPM8 (Ashburner and Friston, 2005), which enables tissue classification, bias correction, segmentation and image registration into a reference stereotaxic space defined by a set of tissue probability templates (Figure 1) (Valdes-Hernandez et al., 2011). Following segmentation, the tissue class images of GM, white matter (WM) and cerebrospinal fluid (CSF) for each rat were used to create a population-specific template (PST) by running the diffeomorphic anatomical registration using exponentiated lie algebra (DARTEL) template-creation tool in SPM8 (Ashburner, 2007). The segmented grey and white matter tissue class images were then spatially and nonlinearly normalized (warped) using the outputs from the previous DARTEL step and modulated by the Jacobian determinant at each voxel to obtain grey and white matter volume maps. The resulting grey and white matter volume maps were then spatially smoothed by convolving them with an isotropic Gaussian kernel of 0.1875 mm full-width at half maximum. This small smoothing kernel has been previously shown to be most effective for the subsequent non-parametric permutation tests (Smith and Nichols, 2009).

Finally, we selected the 30 brain regions with most significant changes by performing a region of interest (ROI)-based extraction of q-values on the resulting SPM map. The extraction was done using the MarsBaR toolbox (Brett et al., 2002) and 154 (77 in each hemisphere) digitized cortical and subcortical structures spanning most of the brain. Within each ROI the most significant voxel was determined and the 30 brain regions with the most significant voxels were reported. The ROIs were derived from the Gaser rat brain atlas (Gaser et al., 2012), which is located in the space of the Paxinos atlas (Paxinos and Watson, 1997). To transfer the ROIs to our study rat brain template (Valdes-Hernandez et al., 2011), the Gaser rat brain atlas was nonlinearly normalized to it using SPM8's normalization tool. Figure 2 shows the rat brain parcellation scheme chosen for the ROI-based q-value extraction. After completion of the VBM analysis, the obtained SPM threshold maps (in the PST space)

containing significant voxels of GMV and white matter volume (WMV) changes were nonlinearly normalized to the rat brain template using nearest neighbour interpolation. This way, the threshold maps and the ROIs were located in the same spatial space.
Note, the terms GM, WM and CSF refer to tissue classifications in the MR images. In particular, some subcortical parts of the brain including the thalamus, which are not strictly WM are classified as such due to their appearance in the MR images, an issue that has previously been raised for the priors used in this study (Valdes-Hernandez et al., 2011, Suzuki et al., 2013, Otte et al., 2015). Therefore, in this study, WM should be understood as tissues with MR contrast similar to WM such as white matter fibres or subcortical brain areas like the thalamus. The smoothed GM and WM compartments were added to constitute the final volume map for the statistical analysis.

Figure 1

Figure 2

Histological analysis

Tissue collection

Animals were sacrificed by transcardial perfusion using heparinised saline followed by 4% paraformaldehyde (Hitobiotec Corp, Kingsport, TN, USA) immediately after the completion of the MRI. Animals were decapitated and their brains quickly removed and immersed in 4% paraformaldehyde for two days, followed by cryoprotection in 30% sucrose (Sigma-Aldrich) for two days at 4C°. Brains were then sectioned at 30 µm on a freezing microtome (Leica, Wetzlar, Germany) at -20C°. Serial coronal sections were subsequently placed in 12-well plates (Thermo Fisher Scientific, Waltham, MA, USA) in 12 series and stored at -20°C until processed for immunohistochemistry.

Immunohistochemistry

Immunohistochemistry was conducted in ten 6-OHDA rats and 12 sham operated rats. Sections were stained with anti-tyrosine hydroxylase (TH) antibody to mark catecholamine neuron cell bodies and their axons. Free-floating sections were pre-treated for 15 min with 1% H_2O_2 (Sigma-Aldrich), washed three times with Tris-buffered saline (TBS; Sigma-Aldrich), blocked with normal goat serum (1:10; Vector Laboratories, Burlingame, CA) and 0.1% Triton-X 100 (Sigma-Aldrich) for 40 min, and then incubated overnight at 4°C with primary antibody (rabbit anti-TH, 1:3000, monoclonal; Chemicon, Temecula, CA, USA). The sections were then washed three times with TBS and incubated with biotinylated goat anti-rabbit (1:1000; Vector Laboratories) secondary antibody for 120 min at room temperature. After three rinses in TBS, the sections were incubated for one hour at room temperature with peroxidase-conjugated avidin–biotin complex kit (Vector Laboratories), washed three times with TBS, and then incubated with 0.02% diaminobenzidine (DAB; Vector Laboratories) until staining was visible. The mounted sections were dehydrated using 100% methylated spirit (Thermo Fisher Scientific) and embedded in DePeX mounting medium (Serva, Heidelberg, Germany).

2.6.3 TH+ fibre density measurements

Due to problems with brain sectioning, one further 6-OHDA rat was excluded from TH+ fibre density measurements (no cortex) leaving nine 6-OHDA rats and twelve sham rats. Analysis of DA denervation in cortical regions (somatosensory cortex, motor cortex, cingulate cortex) was conducted on the TH stained sections. 60 digital images (ten contralateral, ten ipsilateral per section; three consecutive sections) of each rat for each of the three aforementioned brain regions were captured at 40x magnification using a computerized image analysis set-up (Zeiss Axioscope, Carl Zeiss, Gottingen, Germany). Individual fibres in each micrograph were automatically detected using the threshold option triangle in ImageJ (v1.50b; http://imagej.nih.gov/ij/, U.S. National Institutes of Health, Bethesda, Maryland, USA). Briefly, RGB micrographs were converted to BW 8-bit images, and then subjected to background subtraction for bias correction. Then, bias corrected images were thresholded to only highlight the TH+ fibres and the area fraction was determined. The area fraction is the percentage of pixels in the image that have been highlighted after applying ImageJ's triangle threshold. The TH+ fibre density is therefore expressed as this area fraction.

2.6.4 Optical fractionator cell counts of TH-positive neurons in the SN

To examine the total number of TH-positive (TH+) neurons in the SN, stereological quantification was performed using the optical fractionator probe in Stereo investigator software (v7.0, MBF Bioscience, Chicago, IL, USA) running on a computerized image analysis set-up (Carl Zeiss). This procedure was conducted in ten 6-OHDA and twelve sham rats. TH+ cell counts in the SN were determined in both groups for each hemisphere separately. Every 12^{th} 30 µm section encompassing the full rostrocaudal extent of the SN was systematically sampled using an unbiased counting frame. This resulted in an average of 3-5 sections being selected for the SN. The SN was manually outlined at ×2.5 magnification and counting frames were systematically distributed with known x and y steps throughout the region from a random starting point. Cross sectional area of the counting frame was set to 150x150 μm^2 and superimposed on the field of view by the software. This frame area generated on average 4-6 discrete counts per frame. The sampling grid area was estimated individually for each animal in such a way that it comprises of approximately 15-20 counting frames per section. A guard zone of 0.5 µm thickness was used at the top and bottom of each section

with an optical dissector height of 14 µm. All cell counts were performed under ×40 magnification. The estimates of the total number of TH+ cells in the SN were calculated according to the optical fractionator equation (West, 1999). The coefficients of error were calculated according to Gundersen and colleagues with values <0.10 accepted (Gundersen and Jensen, 1987).

Statistical analysis

All data are expressed as mean ± standard error of the mean (SEM) unless stated otherwise. MRI based voxel-wise image analysis was performed using MATLAB, SPM and FSL. Voxel-wise non-parametric permutation (N=5000 permutations) inference employing FSL tool Randomise and the threshold-free cluster enhancement method as described elsewhere (Smith and Nichols, 2009, Winkler et al., 2014) were used to compare the composite volume maps (GMV and WMV) group differences in 6-OHDA and sham rats. Generally, a non-parametric approach is recommended for studies with a low degree of freedom (~20) (Nichols and Holmes, 2002). For an unpaired two-sample t-test the following formula applies to calculate the degree of freedom in our experiment:

$$n_1 + n_2 - 2 = degrees\ of\ freedom$$

where n_1 is the number of subjects in group one and n_2 is the number of subjects in group two. In this chapter, the group pairing resulted in 22 degrees of freedom. Voxels with a probability value below 0.2 in the corresponding priors were excluded to include only voxels with sufficient grey or white matter proportion (May et al., 2007). This diminishes possible edge effects between grey matter, white matter or CSF. A voxel was considered significant only if it exceeded the statistical threshold ($q<0.05$), whereby correction for multiple comparison was done by using the family-wise error rate (FWER). Statistical analysis of the differences in TH+ cells and fibres were conducted using Graphpad Prism (v5.00; GraphPad Software, La Jolla, CA, USA, www.graphpad.com). A two-way ANOVA with Bonferroni post hoc analysis was conducted on the absolute number of TH+ nigral neurons with group as between-subjects factor and hemisphere as within-subjects factor. The TH+ fibre density in cortical parts of the brain was compared using two-way ANOVA (Bonferroni post hoc) with group and hemisphere as main factors. TH+ fibre density were shown as percent of the pixel area fraction and expressed as mean ± SEM. Correlations between the mean grey matter

probability values inside the S1, M1, C1 and SN ROI, and histology or behaviour (6-OHDA group only), respectively, were evaluated using a paired Pearson's correlation. Statistical significance in this chapter was assumed at $p<0.05$, unless stated otherwise.

Results

Behavioural testing: Apomorphine-induced rotations

Following apomorphine challenge, sham rats (N=12) showed a negligible amount of net contralateral turns, whereas 6-OHDA rats (N=13) displayed on average 215.8 ± 42.4 (mean±SD) contralateral turns. Three 6-OHDA-lesioned rats did not reach the cut-off of at least 100 net contraversive rotations and were thus excluded from further experiments.

Voxel-based morphometry reveals grey matter volume decreases in cortical and subcortical brain regions in 6-OHDA-lesioned rat

We next sought to determine whether unilateral lesioning of the MFB leads to MRI detectable grey and white matter volume (GMV and WMV, respectively) changes. Figure 3 shows coronal brain slices of the population specific template (PST) highlighting voxels where GMV differed significantly (q=0.05; FWER-corrected) in 6-OHDA rats compared to shams. The principle findings are that large clusters of significant GMV decrease were found bilaterally in amygdala, cingulate cortex, motor cortex and somatosensory cortex. Additionally, significant voxels of GMV decrease were found in the entorhinal cortex, temporal cortex, dorsal striatum, hippocampus, SN and thalamus of the ipsilateral (lesioned) hemisphere. To precisely localize these GMV changes an atlas-based approach was employed. See Table 1 for a summary of the30 ROIs containing the most significant voxels. The main finding was that brain regions containing the most significant voxels of reduced GMV ($q<0.05$, FWER corrected) were primarily located in the ipsilateral hemisphere of the 6-OHDA lesioned animals, notably in the cortex. There were no significant clusters of increased GMV in 6-OHDA-lesioned animals compared to sham controls.

Significant clusters of WMV loss were also present in the ipsilateral thalamus, a structure that is primarily composed of grey matter but classified as WMV for the purpose of VBM analysis. No other changes in the WMV maps were observed.

Figure 3

Table 1

Post-mortem examination confirms cortical dopaminergic denervation

Qualitative inspection of the immunohistological profile of TH in 6-OHDA rats revealed an almost complete loss of TH+ neurons in the SN (Figure 4A), which is associated with a marked striatal dopaminergic denervation (Figure 4B). To quantitatively determine the extent of the nigral degeneration, we counted the number of TH+ neurons in the SN in both groups. Table 2 shows the neuronal counts in sham and 6-OHDA rats. Two-way ANOVA of TH+ cell counts revealed a main effect of group: $F(1,40)=10.52$, $p=0.0024$; hemisphere: $F(1,40)=38.95$, $p<0.0001$ and group x hemisphere interaction: $F(1,40)=18.21$, $p=0.0001$). *Post hoc* Bonferroni analysis confirmed a significant ($p<0.0001$) reduction in TH+ cells in the ipsilateral substantia nigra of 6-OHDA rats (615.30±114.91) compared to sham operated rats.

Figure 4

Table 2

Further qualitative examination of the 6-OHDA lesioned rat brains revealed markedly reduced dopaminergic innervation in cortical brain areas ipsilateral to the lesion, notably in the motor, cingulate and somatosensory cortices (Figure 5). We therefore next sought to determine the density of TH+ fibres in the cortex. Density values (expressed as % area fraction) of TH+ fibres were significantly different between sham and 6-OHDA animals in the ipsilateral (but not contralateral) cingulate, motor and somatosensory cortices, according to two-way ANOVA (with the main effect of group: cingulate cortex, $F(1,38)=16.23$, $p=0.0003$; motor cortex, $F(1,38)=15.85$, $p=0.0003$ and somatosensory cortex, $F(1,38)=66.77$, $p<0.0001$). Bonferroni post hoc analyses showed that cortical TH+ fibre density in 6-OHDA rats was significantly ($p<0.0001$) reduced in the cingulate, motor and somatosensory cortices (Table 3).

Figure 5

Table 3

To investigate the relationship between grey matter volume changes and TH+ immunoreactivity in these cortical regions, mean grey matter probability values inside the S1, M1 and C1 ROI were correlated with the density of TH+ fibres in 6-OHDA and sham-operated rats (Table 4). There were no significant correlations between the density of TH+ fibres and the mean grey matter probability inside the S1, M1 and C1, or between the TH+ neurons and the mean grey matter probability values in the lesioned SN (Table 4). We also examined and did not detect any significant correlations between the rotational behaviour after apomorphine injection and the mean grey matter probability values inside the S1, M1, C1 and S2 in 6-OHDA rats (Table 5).

Table 4

Table 5

Discussion

The principle findings of this study are that VBM analysis detected specific patterns of significant GMV decreases in several subcortical regions of the ipsilateral (lesioned) hemisphere, including the site of the primary lesion following 6-OHDA lesioning. This pattern of GMV decreases extended to several cortical areas of the cortex, bilaterally in both hemispheres. No significant GMV increases were noted. Focusing on the cortex, we observed that the sites of GMV decrease also showed a marked denervation of the dopaminergic (TH positive) fibres, but these did not correlate with the observed volume changes in these regions.

Structural alterations in the 6-OHDA rat are both similar and distinct to other neurotoxin models of Parkinsonism

Several prior structural MRI have been conducted on 6-OHDA lesioned rats (Van Camp et al., 2009, Van Camp et al., 2010, Soria et al., 2011, Florio et al., 2013), although none tested for the presence of widespread structural changes utilising a whole brain morphometry method. Indeed, prior structural MRI studies in this model have focused on identifying changes in MRI parameters (volume, diffusion or relaxation time) within *a priori* selected ROIs such as the substantia nigra or the striatum. Using this approach, Florio et al. (2013) reported shrinkage of the ipsilateral hemisphere in 6-OHDA rats, which is in agreement with our observation of GMV decrease in several brain regions within the ipsilateral hemisphere. In addition, bilateral trend-level changes in diffusion weighted imaging data are reported in the cortex of 6-OHDA lesioned rats (Soria et al., 2011) echoing the bilateral GMV changes in the motor and cingulate cortices observed in our study.

Prior studies have utilised a similar, but subtly different automated morphometry method (deformation-based morphometry) for whole brain MR image analysis in a proteasome inhibitor (lactacystin) lesioned rat model of PD (Vernon et al., 2010, Vernon et al., 2011). Here, the lactacystin is infused into the left-MFB, leading to widespread brain pathology. Despite some important differences between the neurotoxic mechanisms in the two models, there is a striking overlap in the MR imaging findings. In particular, lactacystin rats show cortical, striatal and thalamic GMV atrophy in the lesioned hemisphere (Vernon et al., 2011, Harrison et al., 2015), all of which were also detected in the 6-OHDA rats. Of note is that the

clusters of cortical changes were also extending into the contralateral (non-lesioned) hemisphere. In contrast, prior studies in the lactacystin-lesioned rat did not find significant volume changes in the hippocampus, perhaps due to the small sample size compared to the current study (Vernon et al., 2010, Vernon et al., 2011). Furthermore, by three weeks post-lesion lactacystin also induced visible deformation of the ventral midbrain, extending beyond the substantia nigra, which worsened with increasing time post-lesion and was due to extensive neuronal loss (Vernon et al., 2011, Harrison et al., 2015, Pienaar et al., 2015). In the current study, 6-OHDA lesioned rats did display significant GMV atrophy in the hippocampus and did not show extensive GMV atrophy in the midbrain, but rather, restricted nigral atrophy. It should also be noted that we were not able to also confirm ventricular enlargement in the 6-OHDA rat, which could be explained by the limitation of VBM which only uses grey and white matter segments for the analysis. Since only a single-time point was examined in the current study, we cannot exclude the possibility that such changes would emerge in the 6-OHDA model at longer time-points post-lesion. Nevertheless, these data suggest some important commonalities but also differences in the two model systems, which may be expected given the potential for non-selective toxicity with lactasystin lesions, particularly at higher doses (Vernon et al., 2011, Mackey et al., 2013). Importantly, features such as midbrain atrophy are more characteristic clinically of atypical Parkinsonism such as progressive supranuclear palsy and multiple system atrophy (Paviour et al., 2006). It may therefore be suggested that high-dose lactacystin lesion models have greater face validity to atypical forms of Parkinsonism. Further longitudinal studies directly comparing the two models using lower doses of lactacystin that induce more selective lesions may however be informative in this respect (Mackey et al., 2013).

Comparison to clinical findings

Interestingly, many of the brain regions we found to have decreased GMV in the 6-OHDA rats correspond to the analogous brain regions affected by PD in humans, according to a recent voxelwise meta-analysis of clinical VBM studies (Shao et al., 2015). In particular, atrophy of the prefrontal, temporal, occipital and parietal cortices can be generally observed even in early stage PD (Borghammer et al., 2010, Lyoo et al., 2010, Tessa et al., 2014), but is more pronounced in advanced PD, specifically in the temporal brain areas of PD patients with

dementia (Burton et al., 2004, Song et al., 2011, Weintraub et al., 2011, Hattori et al., 2012, Melzer et al., 2012). As can be appreciated from these studies, cortical atrophy has become a defining feature of both idiopathic PD and PD with dementia, and here we supply evidence of the similar MRI phenotype in the 6-OHDA rat model.

Structure-function relationships

Our preclinical data confirm the clinical findings and earlier pre-clinical data from other models (e.g. (Vernon et al., 2011)), which suggest that *cortical* GMV decrease is a prominent consequence of the nigrostriatal degeneration. This notion is strengthened by the evidence of cortical *functional* changes following 6-OHDA lesioning. Specifically, using positron emission tomography imaging (Casteels et al., 2008) and 2-deoxy-D-glucose autoradiography (Carlson et al., 1999) researchers have revealed a decreased glucose metabolism in the sensorimotor cortex, and by electroencephalography Sharott and colleagues have observed reduced neural firing also in the sensorimotor cortex (Sharott et al., 2005). It is however not clear whether the decreased metabolism and neural firing are causes or consequences of the reduced volume of cortical grey matter.

Of interest is a previous study that demonstrated a positive correlation between diminished cerebral blood volume response to a nociceptive stimulus and dopaminergic denervation in the striatum in 6-OHDA rats (Chen et al., 2013). Although speculative, it is conceivable that cortical dopaminergic denervation may affect cerebral blood volume response in the cortex in a similar fashion, which may be further followed-up using resting-state functional MRI to establish a structure-function relationship.

It was beyond the scope of this project to perform extensive behavioural measurements which have already been well characterised by others (Schwarting and Huston, 1996, Deumens et al., 2002). Thus, we were not able to *directly* explore the association between MRI-derived structural changes and parkinsonian behavioural deficits. Historically, 6-OHDA lesion was deemed to be restricted to the nigrostriatal system and therefore the most frequently conducted behavioural tests are for motor activity and coordination, which have indeed shown a good correlation between the nigrostriatal degeneration and motor defects in tasks such as accelerating rotarod (Monville et al., 2006, Carvalho et al., 2013) and adjusting stepping test (Olsson et al., 1995, Decressac et al., 2012).

In light of our cortical atrophy findings, however, it would be of future interest to determine the relative contribution of the cortical vs. subcortical structural changes to the different aspects of motor and cognitive behavioural deficits in this model. It may be hypothesised that cortical damage additionally contributes to both motor as well as sensory deficits in the 6-OHDA rat. It has been shown, for example, by Montoya and colleagues that a unilateral lesion of the rat sensorimotor cortex leads to a clear motor impairment (Montoya et al., 1991) underscoring the contribution of the cortex to movement. Moreover, another study showed specific memory and learning deficits in 6-OHDA rats (Dolatshahi et al., 2015), which are in line with the widespread GMV loss we observed in the prefrontal cortex, a region important for cognitive function that is also known to be affected in human PD patients. Additionally, the aforementioned lactacystin rat study found that the thinning of the motor cortex, rather than nigrostriatal changes, best predicted the degree of motor deficits (Vernon et al., 2011). The degree to which cortical changes influence cognitive (dys)function in 6-OHDA rat remains to be confirmed given that several other studies did not find any evidence of reduced cognitive abilities in this model (Mura and Feldon, 2003, Carvalho et al., 2013).

The neurobiological correlate of grey matter changes is diverse

The interpretation of the VBM results as tissue volume changes are based on significant differences in MRI contrast, which can also be governed by the changes in tissue properties. For example, a recent VBM study in rodents reported a significant correlation of VBM signal with changes in spine density (Keifer et al., 2015), and another VBM study in a rat model of multiple sclerosis found evidence for changes in dendrite morphology in the sensorimotor cortex alongside GM atrophy (Tambalo et al., 2015). Similarly, in a rat model of schizophrenia, MRI-derived GM atrophy was suggested to be associated with gross neuronal loss and dendrite plasticity (Wu et al., 2016) highlighting the effect of microscale changes on macroscopic morphology.

We speculate that both gross neuronal loss in the substantia nigra and the ensuing dopaminergic denervation contributed to MRI-derived GM atrophy in 6-OHDA rats. In particular, we showed a remarkable overlap of GMV loss in cingulate, motor and somatosensory cortex on the lesioned side with a significant TH+ fibre denervation in the same areas in 6-OHDA rats. TH is a marker of catecholamine neurons, which include DA and

noradrenergic neurons. Two previous 6-OHDA studies, in which noradrenergic neurons were protected by desipramine prior to the lesioning surgery showed a similar cortical denervation of TH+ fibres (Debeir et al., 2005, Ueno et al., 2014) suggesting that the loss of TH+ fibres in the cortex is DA-specific.

Furthermore, as expected after 6-OHDA MFB lesioning, we found a significantly reduced number of dopaminergic neurons in the lesioned SN and the marked DA denervation of the striatum. Notably, both grey matter loss in the SN and the striatum were successfully detected using VBM analysis, too. Our histopathological examinations of the cortex are supported by, and extend previous reports of DA fibre loss in the ipsi-lesional cingulate and motor cortices in the 6-OHDA rats (Debeir et al., 2005, Castillo-Gomez et al., 2008, Hou et al., 2010, Ueno et al., 2014).

We speculate that the loss of DA fibres in the cortex and elsewhere indirectly underlies changes observed by MRI. MRI measurements are particularly sensitive to changes in water proton properties, which can be driven by diverse cellular and extracellular processes (Zatorre et al., 2012, Sumiyoshi et al., 2014) such as neurogenesis, synaptogenesis, changes in neuronal and glial morphology as well as extracellular space and inflammation (Eriksson et al., 2009, Lerch et al., 2011, Finlay et al., 2014). With respect to this, DA denervation in the cortex and striatum has been shown to lead to a structural remodelling of the surrounding tissue such as altered wiring of intrinsic inhibitory GABA-ergic circuits (Solis et al., 2007, Castillo-Gomez et al., 2008), reduced synaptic density (Castillo-Gomez et al., 2008, Hou et al., 2010), spine enlargement (Ueno et al., 2014) and loss of glutamatergic input from the prefrontal cortex to the medium spiny neurons of the striatum (Day et al., 2006, Solis et al., 2007), all of which are likely to contribute to changes in the water proton properties ultimately modulating MRI signal. Given the lack of correlation between MRI and histological measures, it is likely that the dopaminergic denervation triggers a cascade of events that eventually lead to microscale tissue reorganization, which then ultimately underlie the observed MRI signal changes.

Bilateral brain changes are a distinctive feature of the unilateral 6-OHDA rat model

It is of note that we found widespread changes in both sides of the brain in this unilaterally lesioned rat model. This supports previous findings reporting bilateral structural, physiological and functional changes in the 6-OHDA rat brain (Morgan and Huston, 1990,

Pelled et al., 2002, Breit et al., 2008, Pierucci et al., 2009, Van Camp et al., 2010, Soria et al., 2011, Viaro et al., 2011). For example, Pelled and colleagues described a bilateral overactivation of the somatosensory cortex after a unilateral electrical forepaw stimulation (Pelled et al., 2002). Another study reported bilateral changes in water diffusion properties as measured by diffusion-weighted imaging in the SN and the cortex (Soria et al., 2011). Using intracortical microstimulation, Viaro et al. reported bilaterally reduced M1 excitability (Viaro et al., 2011). Together with our findings, these results suggest that the mutual influence of both hemispheres in the pathophysiology of the unilateral 6-OHDA rat is an important and defining feature of this model.

Utility of VBM in 6-OHDA rats

The rationale for using VBM in this study was to define an MRI phenotype based on a technique that is now widely used in clinical research. Given the paucity of analogous studies in 6-OHDA rats, our present work establishes the first MRI phenotype based on VBM image analysis and therefore helps to bridge the gap between clinical and preclinical studies. Although further work is required to elucidate the mechanisms that underlie the cortical grey matter loss in this rat model, the MFB 6-OHDA rat model might be useful for studying the controversial effect of DA replacement therapies in humans (Finlay et al., 2014). Indeed, previous reports have found evidence of modulating effects of levodopa therapy on both the cortical MRI signal in healthy subjects (Salgado-Pineda et al., 2006) and in dyskinetic PD patients (Cerasa et al., 2013) and the 6-OHDA rats model may be a suitable model to investigate the relationship between cortical brain changes and levodopa treatment.

Conclusion

We have shown that VBM can be readily applied to rats for morphometric assessment of 6-OHDA lesion induced changes. We found VBM particularly useful to localize the large clusters of grey matter loss in the cortex, which matched the areas of DA fibre loss. This shows the potential of automated image analysis to advance the current preclinical MRI research by revealing new and unexpected sites of neurodegeneration beyond the primary lesioned pathway. Additionally, the changes were similar to those described in other rat models and in the human advanced PD, reinforcing not only the value of the MFB 6-OHDA rat model as a model of late-stage PD, but also opening up avenues toward utilising GMV in animals as proxy marker of (neuro)degeneration in humans. These data thus reinforce the power and utility of combining MRI and clinically relevant imaging analysis tools for the evaluation of rodent models of Parkinsonism. We therefore propose that this established framework should be applied as standard to emerging PD rat models that show slower and progressive neurodegeneration such as the recombinant adeno-associated viral vector overexpressing alpha-synuclein rat (Decressac et al., 2012, Van der Perren et al., 2014) and transgenic mouse models (Janezic et al., 2013), which ultimately have greater construct and face validity. For example, this would facilitate the correlation of morphological phenotypes with the onset of behavioural deficits in a longitudinal fashion, which may ultimately increase the chance of identifying predictive biomarkers for early-onset PD, response to therapy and disease progression and establish at the cellular level the mechanisms which drive MRI signal changes in PD patients.

Conflict of interest statement

None of the material contained in this manuscript has been published or presented previously, except in abstract form at international conferences. This paper has not been submitted for publication elsewhere, and it has been reviewed and approved by all the authors. There are no financial or other relationships that could lead to a conflict of interest.

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Figures and Tables Legend

FIG1

Tissue probability maps for (A) grey matter, (C) white matter and (D) CSF in the living rat brain.

FIG2

Rat brain parcellation scheme. Rat brain was segmented into 154 ROIs (77 on each hemisphere). Each bilateral ROI is labelled differently and overlaid onto coronal slices of the rat brain template.

FIG3

Voxel-wise differences in local grey matter between the 6-OHDA and sham group three weeks post-lesioning. Absolute regional grey and white matter volume maps were computed and non-parametric permutation two-sample t-test was applied at every voxel to identify significant grey and white matter volume changes. Statistical parameter maps were overlaid on the population-specific template as anatomical reference. The colour calibration bar represents the q-values with a threshold level of $q<0.05$ (FWER corrected). Increased regional volume in 6-OHDA rats compared to shams was not observed, decreased regional volume is displayed in blue. Note, no true white matter changes were observed. L, lesioned side; R, unlesioned side. 6-OHDA rats N=13; sham rats N=11.

FIG4

Pattern of TH immunoreactivity in a representative 6-OHDA rat. DAB immunolabelling showing the location of TH+ neurons in the contralateral SN (A) and fibres in the contralateral striatum (B) but not ipsilateral, as indicated by the asterisks. L, ipsilateral, lesioned side; R, contralateral, unlesioned side.

FIG5

Tyrosine hydroxylase immunoreactivity in sections through prefrontal and frontal cortex from a representative 6-OHDA rat. (A) Primary motor cortex and the magnified areas in the (D) contralateral and (E) ipsilateral hemisphere. (B) Cingulate cortex and the magnified areas in the (F) contralateral and (G) ipsilateral hemisphere. (C) Somatosensory cortex and the magnified areas in the (H) contralateral and (I) ipsilateral hemisphere. Overall, the ipsilateral cortex was found to have a profound loss of TH+ fibres, which was associated with dopaminergic striatal denervation as evidenced by the absence of TH+ immunoreactivity in the ipsilateral striatum. White frames indicate the areas with higher magnification (x40). Scale bar = 100 µm in I (applies to D-I). L, ipsilateral, lesioned side; R, contralateral, unlesioned side.

TAB1

Results of the ROI-based localization analysis of the 30 ROIs containing the most significant voxels of regional grey matter volume decrease three weeks after 6-OHDA lesioning. Arranged in descending order from lowest to highest q-value found in each brain region. Voxels thresholded at $q<0.05$, FWER corrected. L, ipsilateral 6-OHDA lesioned hemisphere; R, contralateral unlesioned hemisphere. H, hemisphere; T, total number of voxels within the brain region; S, total number of significant voxels within each brain region; S/T, percentage of voxels that are significant within the brain region; Q, smallest q-value of a voxel found inside a ROI.

TAB2

Stereological estimates of nigral TH+ neuronal numbers in sham and 6-OHDA rats. Neuronal counts of TH+ cells were significantly different between sham and 6-OHDA animals as two-way ANOVA revealed with main effect of group: $F(1,40)=10.52$, $p=0.0024$; main effect of hemisphere: $F(1,40)=38.95$, $p<0.0001$ and group/hemisphere interaction: $F(1,40)=18.21$, $p=0.0001$ (Bonferroni post hoc test 6-OHDA ipsilateral vs. sham ipsilateral ****$p<0.0001$). Data are expressed as mean cell count ± SEM. 6-OHDA rats N=10; sham rats N=12.

TAB3

Comparison of TH immunoreactivity of cortical dopaminergic fibres in 6-OHDA and sham rats. TH+ fibre density was significantly different between sham and 6-OHDA animals in the ipsilateral but not contralateral hemisphere as two-way ANOVA revealed with main effect of group: cingulate cortex, $F(1,38)=16.23$, $p=0.0003$; motor cortex, $F(1,38)=15.85$, $p=0.0003$; somatosensory cortex, $F(1,38)=66.77$, $p<0.0001$. (Bonferroni post hoc test revealed significantly reduced TH+ fibre density in ipsilateral cingulate, motor and somatosensory cortices in 6-OHDA rats compared to shams, ****$p<0.0001$). Data are shown as percent of the area fraction of TH+ fibre pixels and expressed as mean ± SEM. 6-OHDA rats N=9; sham rats N=12. CC, cingulate cortex; MC, motor cortex; SC, somatosensory cortex.

TAB4

Correlation of the immunohistological profile of TH and grey matter volume changes in four regions of interest. Correlation analyses were done using the Pearson's correlation coefficient. No significant correlation was found. Numbers in brackets indicate the correlation coefficient for sham rats. TH, tyrosine hydroxylase; GMV, grey matter volume; C1, primary cingulate cortex; M1, primary motor cortex; S1, primary somatosensory cortex; SN, substantia nigra. 6-OHDA rats N=9, sham rats N=11.

TAB5

Correlation of apomorphine-induced rotations the immunohistological profile of TH and grey matter volume changes in four regions of interest. Correlation analyses were done using the Pearson's correlation coefficient. No significant correlation was found. GMV, grey matter volume; C1, primary cingulate cortex; M1, primary motor cortex; S1, primary somatosensory cortex; SN, substantia nigra. 6-OHDA rats N=9.

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